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Sarcomeric perturbations of myosin motors lead to dilated cardiomyopathy in genetically modified *MYL2* mice

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Dilated cardiomyopathy (DCM) is a devastating heart disease that affects about 1 million people in the United States, but the underlying mechanisms remain poorly understood. In this study, we aimed to determine the biomechanical and structural causes of DCM in transgenic mice carrying a novel mutation in the *MYL2* gene, encoding the cardiac myosin regulatory light chain. Transgenic D94A (aspartic acid-to-alanine) mice were created and investigated by echocardiography and invasive hemodynamic and molecular structural and functional assessments. Consistent with the DCM phenotype, a significant reduction of the ejection fraction (EF) was observed in ∼5- and ∼12-mo-old male and female D94A lines compared with respective WT controls. Younger male D94A mice showed a more pronounced left ventricular (LV) chamber dilation compared with female counterparts, but both sexes of D94A lines developed DCM by 12 mo of age. The hypocontractile activity of D94A myosin motors resulted in the rightward shift of the force–pCa dependence and decreased actin-activated myosin ATPase activity. Consistent with a decreased Ca^{2+} sensitivity of contractile force, a small-angle X-ray diffraction study, performed in D94A fibers at submaximal Ca^{2+} concentrations, revealed repositioning of the D94A cross-bridge mass toward the thick-filament backbone supporting the hypocontractile state of D94A myosin motors. Our data suggest that structural perturbations at the level of sarcomeres result in aberrant cardiomyocyte cytoarchitecture and lead to LV chamber dilation and decreased EF, manifesting in systolic dysfunction of D94A hearts. The D94A-induced development of DCM in mice closely follows the clinical phenotype and suggests that *MYL2* may serve as a new therapeutic target for dilated cardiomyopathy.

MYL2 | myosin RLC | DCM | transgenic D94A mice | invasive hemodynamics

Dilated cardiomyopathy (DCM) is a heart disease characterized by left ventricular (LV) dilation, normal or reduced LV wall thickness, interstitial fibrosis, and significantly diminished contractile function (1). It is one of the most common cardiomyopathies, with a predicted occurrence of 1 in 400 in the United States (2). DCM may be sporadic and caused by ischemia, alcohol toxicity, or viral infection, or it can be genetic and arise from mutated genes. Inherited gene defects account for up to 25% of cases of DCM and include mutations in more than 40 different genes, including cytoskeletal, sarcomeric, sarcolemmal, and nuclear envelope proteins (3). In the family of sarcomeric proteins, mutations in at least nine genes encoding for myosin heavy chain (MHC), β-MHC (*MYH7*), α-MHC (*MYH6*), MyBP-C (myosin-binding protein C), titin, cardiac actin, troponin (Tn) complex, and α-Tm (tropomyosin) proteins are implicated in DCM (4). Recently, our group reported on a mutation in *MYL2*, encoding the myosin regulatory light chain (RLC), identified by exome sequencing in a pedigree with familial DCM (5). Biochemical studies of the recombinant aspartic acid-to-alanine RLC mutant (D94A-RLC) showed that subtle changes in the secondary structure of the RLC and its interaction with the MHC resulted in chemomechanical uncoupling and malfunction of D94A-mutated myosin motor protein (5).

Myosin RLC is a major regulatory subunit of striated-muscle myosin and a modulator of Ca^{2+} and tropomyosin–troponin–controlled regulation of cardiac muscle contraction (6). It is localized at the head–rod junction of the MHC and, together with the myosin essential light chain, stabilizes the α-helical neck region of the myosin head, also called the lever arm (7). To address the mechanisms responsible for triggering abnormal function of D94A-RLC myosin and cardiac defects consistent with DCM, we have generated transgenic (Tg) mice expressing the D94A-mutated human ventricular RLC. Age- and sex-matched Tg-WT (wild-type) mice, expressing human ventricular RLC and produced in this laboratory previously (8), served as controls. The Tg-D94A animal model is a clinically relevant rodent model of human familial DCM with a mutation in myosin RLC encoded by *MYL2* (5).

We provide strong evidence that the D94A mutation in cardiac myosin RLC controls contractility via structural alterations of myosin thick filaments and their interaction with the regulated actin filaments triggering an aberrant cross-bridge cycling and down-regulating myofilament response to Ca^{2+}. These changes ultimately result in significant reduction of the ejection fraction (EF) and progressive dilated cardiomyopathy in D94A-mutated

Significance

Dilated cardiomyopathy (DCM) is a progressive heart disease with no current cure, often culminating in heart transplantation. Transgenic D94A (aspartic acid-to-alanine) mice carrying a novel DCM-causative mutation in the *MYL2* gene, encoding the cardiac myosin regulatory light chain, were created and investigated by echocardiography and invasive hemodynamic and molecular structural and functional assessments. Our data show that hypocontractile myosin motors and structural perturbations at the level of sarcomeres trigger aberrant functional remodeling in D94A hearts and the development of DCM, which closely follows the clinical phenotype. Left ventricular chamber dilation and decreased ejection fraction, observed in D94A hearts, were indicative of systolic dysfunction, a hallmark of DCM. Our study suggests that *MYL2* may be considered a therapeutic target for dilated cardiomyopathy.

Author contributions: C.-C.Y. and D.S.-C. designed research; C.-C.Y., K.K., J.L., Z.Z., and S.Y. performed research; A.V.G. and T.C.I. contributed new reagents/analytic tools; C.-C.Y., K.K., J.L., Z.Z., S.Y., A.V.G., T.C.I., and D.S.-C. analyzed data; and D.S.-C. wrote the paper.

The authors declare no conflict of interest.

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animals compared with respective WT-RLC control mice. Our in-depth study clearly shows that the *MYL2* variant, with the D94A mutation in the myosin RLC, closely follows the clinical DCM phenotype, providing a foundation for including the *MYL2* gene in the pool of ∼40 currently screened DCM genes (3), and that the myosin ventricular RLC may be considered an additional therapeutic protein target for DCM.

Results

Generation of the D94A-RLC Animal Model of DCM. To address the mechanisms underlying cardiac dysfunction due to the D94A mutation in myosin ventricular RLC protein (UniProtKB P10916), we generated two lines (Ls) of the α-MHC–driven transgenic mice expressing the D94A-mutated human ventricular RLC (Fig. 1*A*). Female (F) and male (M) D94A-L1 and -L2 animals were used in the experiments, and the results were compared with age- and gender-matched Tg-WT-RLC mice (8). Direct assessment of the human (%) versus mouse (%) cardiac RLC in the ventricles of WT mice and in D94A-L1 and D94A-L2 by mass spectrometry showed the expression of 87.6 ± 4.8 versus 12.4 ± 4.9 (WT), 53.4 ± 3.2 versus 46.6 ± 3.2 (L1), and 49.5 ± 2.8 versus 50.5 ± 2.8 (L2) (Fig. 1*B*). Therefore, the generated model of DCM with ∼50% of mutant RLC expression recapitulated the heterozygous transmission of the mutated allele in a D94A-positive family (5). No significant changes in RLC phosphorylation due to the D94A mutation occurred in the hearts of mice, as tested by 2D SDS/PAGE using mouse ventricular preparations (myosin and myofibrils) and RLC-specific antibodies (Fig. 1*C*). The latter result is consistent with our in vitro study showing that the phosphorylation of RLC by Ca^{2+}/calmodulin-activated myosin light chain kinase was not affected by D94A (5).

Ventricular Histology and Ultrastructure of D94A Hearts. Cardiac phenotypes in Tg-D94A lines were identical and showed biventricular cardiac dilation that was prominent by age ∼5 mo in animals of both sexes, as evidenced by gross pathology images and those of longitudinal heart sections of D94A compared with WT mice (Fig. 2*A*). The myocardial ultrastructure assessed by transmission electron microscopy in ∼9-mo-old D94A mice revealed myopathic vacuolar formations in the myocardium of

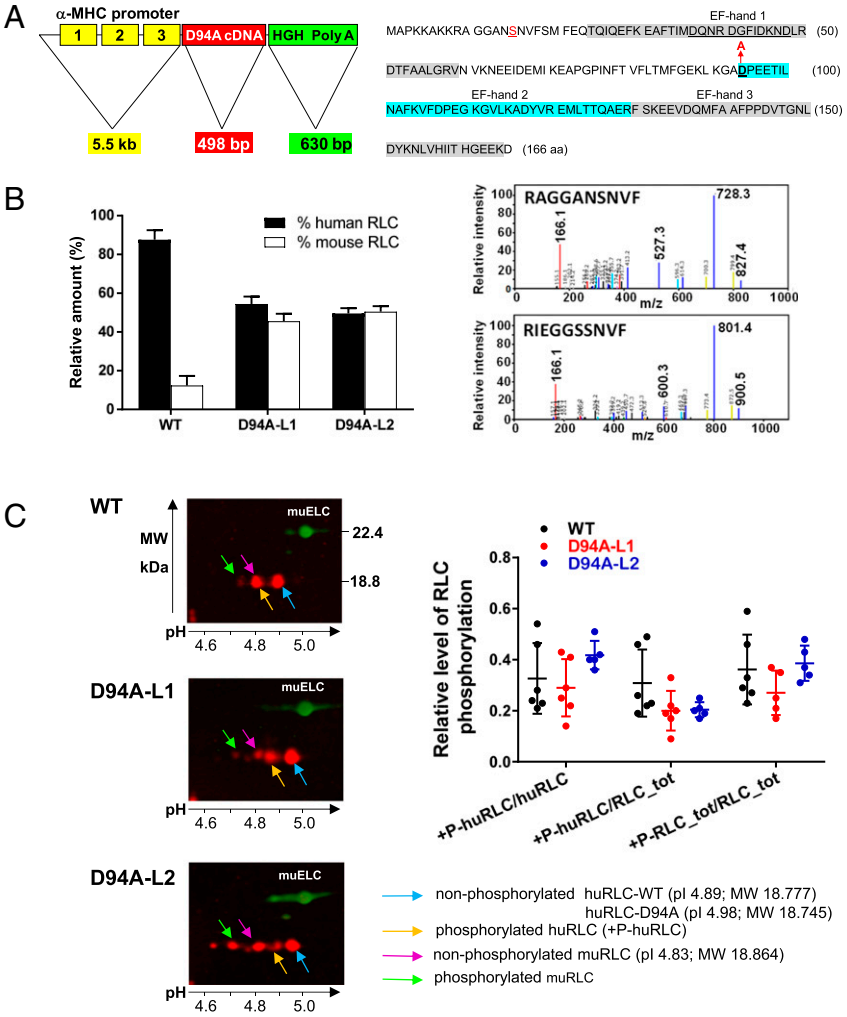


Fig. 1. (*A*) Transgene construct with α-MHC promoter for expression of the D94A-RLC mutant (*Left*) and amino acid sequence of human cardiac RLC UniProtKB P10916 (pI 4.89; molecular mass 18.789 kDa) (*Right*). HGH, human growth hormone. (*B*) Relative expression of D94A-RLC in Tg mice. (*B*, *Right*) Sample spectra of human (*Upper*) and mouse (*Lower*) RLC sequences. (*C*) Two-dimensional SDS/PAGE and Western blots of ventricular myosin from WT, D94A-L1, and D94A-L2 mouse lines. RLC proteins were visualized with the CT-1 antibody and the myosin essential light chain (ELC) protein (used as a loading control) with the monoclonal ab680 antibody (Abcam). Phosphorylated and nonphosphorylated human (huRLC-WT and huRLC-D94A) and mouse (muRLC) RLC proteins were separated according to their respective molecular masses and pI, and were quantitated using $n = 5$ or 6 myosin/myofibrillar preparations per line ±SD. No statistical difference in RLC phosphorylation was observed between WT, D94A-L1, and D94A-L2 mice, $P > 0.05$, using one-way ANOVA.

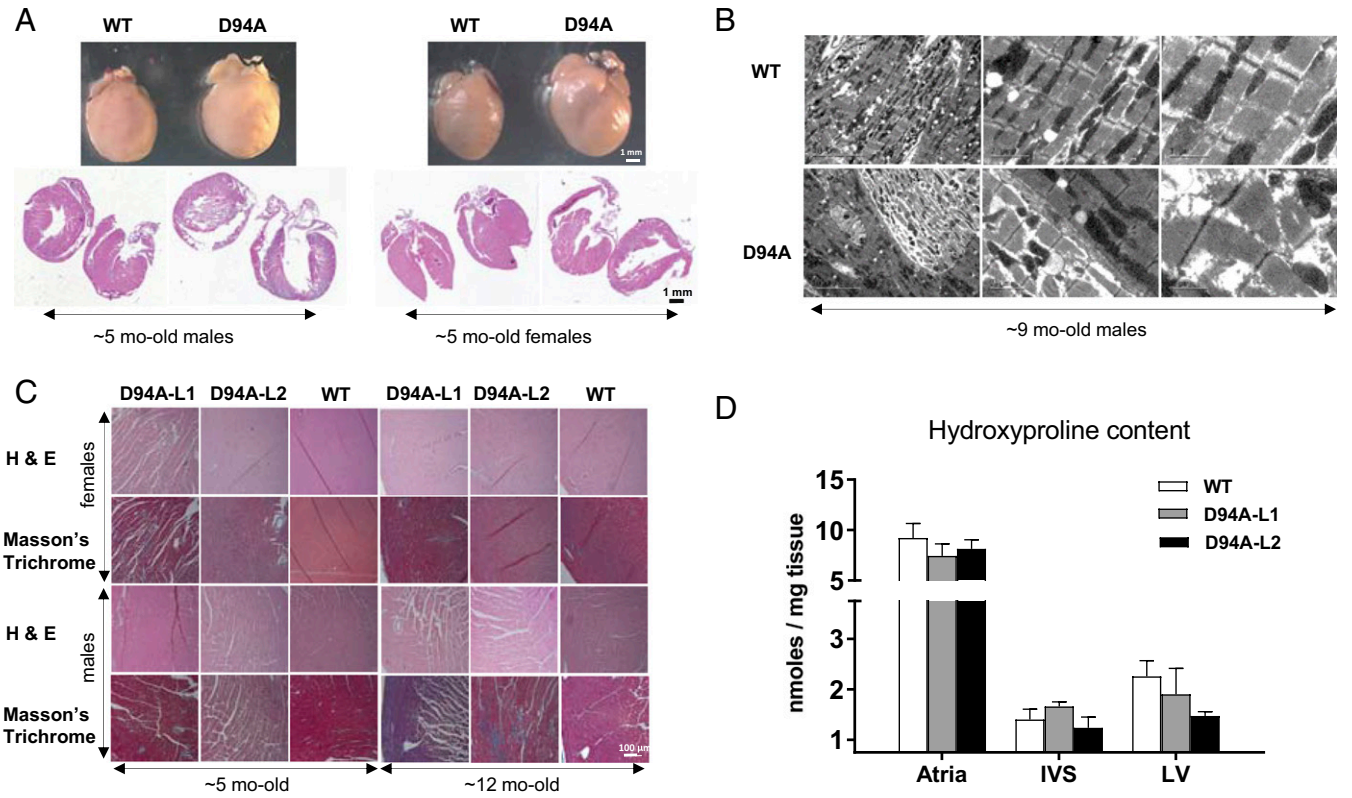


Fig. 2. (*A*) Hearts of representative ~5-mo-old male and female D94A mice and their longitudinal sections showing biventricular cardiac dilation compared with respective WT controls. (Scale bars, 1 mm.) (*B*) Transmission electron microscopy images of ~9-mo-old D94A-L1 vs. WT hearts. [Scale bars, 10 μm (*Left*), 2 μm (*Middle*), and 1 μm (*Right*).] Note excessive vacuolar formations in the myocardium of D94A vs. WT mice. (*C*) Hematoxylin and eosin- and Masson's trichrome-stained LV sections from ~5- and ~12-mo-old M and F D94A-L1 and -L2 vs. WT mice. (Scale bar, 100 μm.) (*D*) Hydroxyproline content in the hearts of 7- to 11-mo-old M and F D94A-L1 and -L2 compared with WT mice.

D94A versus WT mice and disrupted sarcomeric structures in the mutant compared with age- and sex-matched WT-RLC mice (Fig. 2*B*). Histopathology evaluation of left ventricular tissue from ~5- and ~12-mo-old D94A animals showed no obvious myofilament disarray or fibrosis, and the rare fibrotic depositions occurred in the hearts of older male but not female D94A mice (Fig. 2*C*). The lack of fibrosis in D94A mice was confirmed by a quantitative hydroxyproline (HOP) assay, which showed no difference in HOP content in atria, interventricular septum, or LV tissue between D94A lines and WT mice (Fig. 2*D*).

LV Chamber Dilation and Severely Reduced Ejection Fraction in D94A Hearts. As heart chamber size increased, D94A mice developed severely diminished cardiac function, as assessed by echocardiography and invasive hemodynamics (9, 10) (Fig. 3). Consistent with the DCM phenotype, a significant reduction of the ejection fraction and fractional shortening occurred in younger and older D94A-L1 and -L2 mice compared with respective WT controls (Table 1, Table S1, and Movies S1 and S2). A largely dilated left ventricular chamber (increased LVID) was observed in young male D94A mice compared with female counterparts, but both sexes of D94A lines developed progressive dilated cardiomyopathy by 12 mo of age (Fig. 3*A*, Fig. S1, and Table S1).

Systolic Dysfunction in Young and Old D94A Mice. Hemodynamic assessment and pressure–volume loop analysis of D94A versus WT hearts showed significantly decreased intact heart function in both age groups of D94A mice compared with respective WT mice (Fig. 3*B* and Movies S1 and S2). Older D94A mice demonstrated a more pronounced phenotype compared with younger D94A mice, and the diminished cardiac function was more severe in male than in female D94A mice (Fig. S1 and Table S1). Decreased stroke work, cardiac output, and prerecruitable stroke work in D94A versus WT animals were paralleled by significantly elevated end-systolic and end-diastolic volume) compared with respective WT mice (Fig. 3*B*, Table 1, and Table S1).

In addition, D94A animals showed elevated values of Ea (arterial elastance) compared with WT mice that were present in young and old D94A mice of both sexes (Fig. 3*B*). Increased Ea is a sympathetic response to compensate for reduced LV stroke volume, which is controlled by the autonomic nervous system (11) and may be responsible for the D94A-mediated reduction of mechanical efficiency and the ventricular–arterial decoupling (12).

Our combined data indicate that young and old D94A mice of both sexes clearly suffer from systolic dysfunction, and no diastolic disturbance (no changes in the relaxation constant; tau) was observed in 5- or 12-mo-old F and M D94A mice compared with respective WT controls (Fig. 3).

Abnormalities of Myofilament Contractility in Skinned and Intact Papillary Muscle Fibers from D94A Mice. Skinned papillary muscle strips isolated from 5-mo-old D94A mice demonstrated a decrease in the Ca^{2+} sensitivity of force, with ΔpCa_{50} ~0.13 and ~0.25 for D94A mice of both sexes compared with respective WT controls (Fig. 4*A* and Table 2). No significant changes between genotypes were observed in the ability of myosin to develop maximal contractile force and, except for D94A-L1-F, no changes in maximal tension at pCa 4 per cross-section of muscle were noted. A similar trend of decreased Ca^{2+} sensitivity of force with no changes in F_{max} was observed in older animals (Fig. 4*A* and Table 2). Both age groups of mice demonstrated a DCM characteristic rightward shift in the force–pCa dependence and a significantly reduced

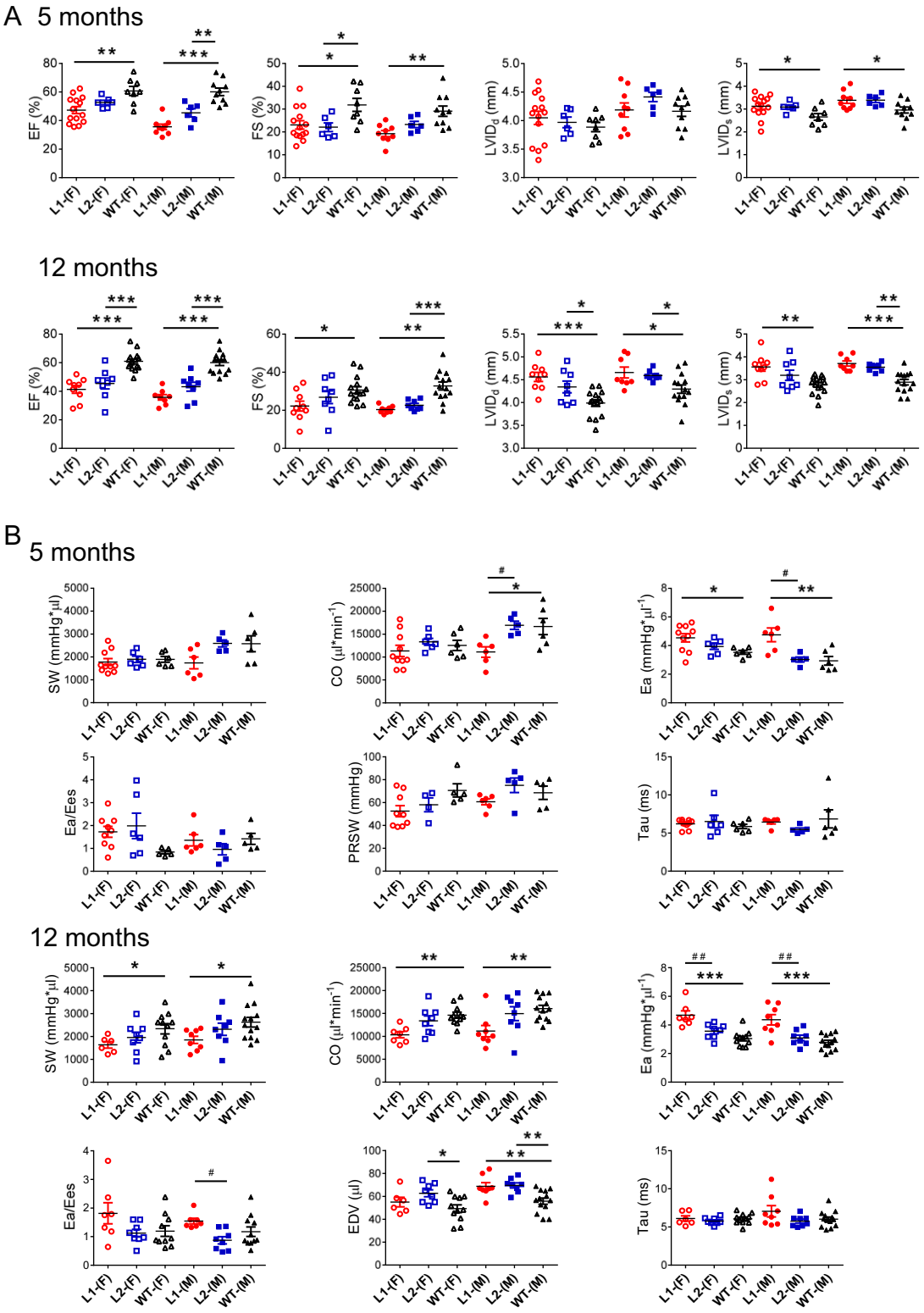


Fig. 3. In vivo characterization of heart morphology and function in D94A vs. WT lines by echocardiography (*A*) and invasive hemodynamics (*B*). Two lines of D94A (L1 and L2) female (open symbols) and male (closed symbols) were used and the results were compared with age- and sex-matched WT mice. CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; FS, fractional shortening; LVID, left ventricular inner diameter in diastole (d) and systole (s); PRSW, prerecruitable SW; SW, stroke work; Tau, relaxation time constant. The number of F and M mice examined is given in Table 1. Data are the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ D94A vs. WT, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ L1 vs. L2, by one-way ANOVA and the post hoc Tukey's multiple comparison test.

Table 1. Intact heart function in ~5-mo-old D94A-RLC mice compared with WT-RLC animals by echocardiography and invasive hemodynamics

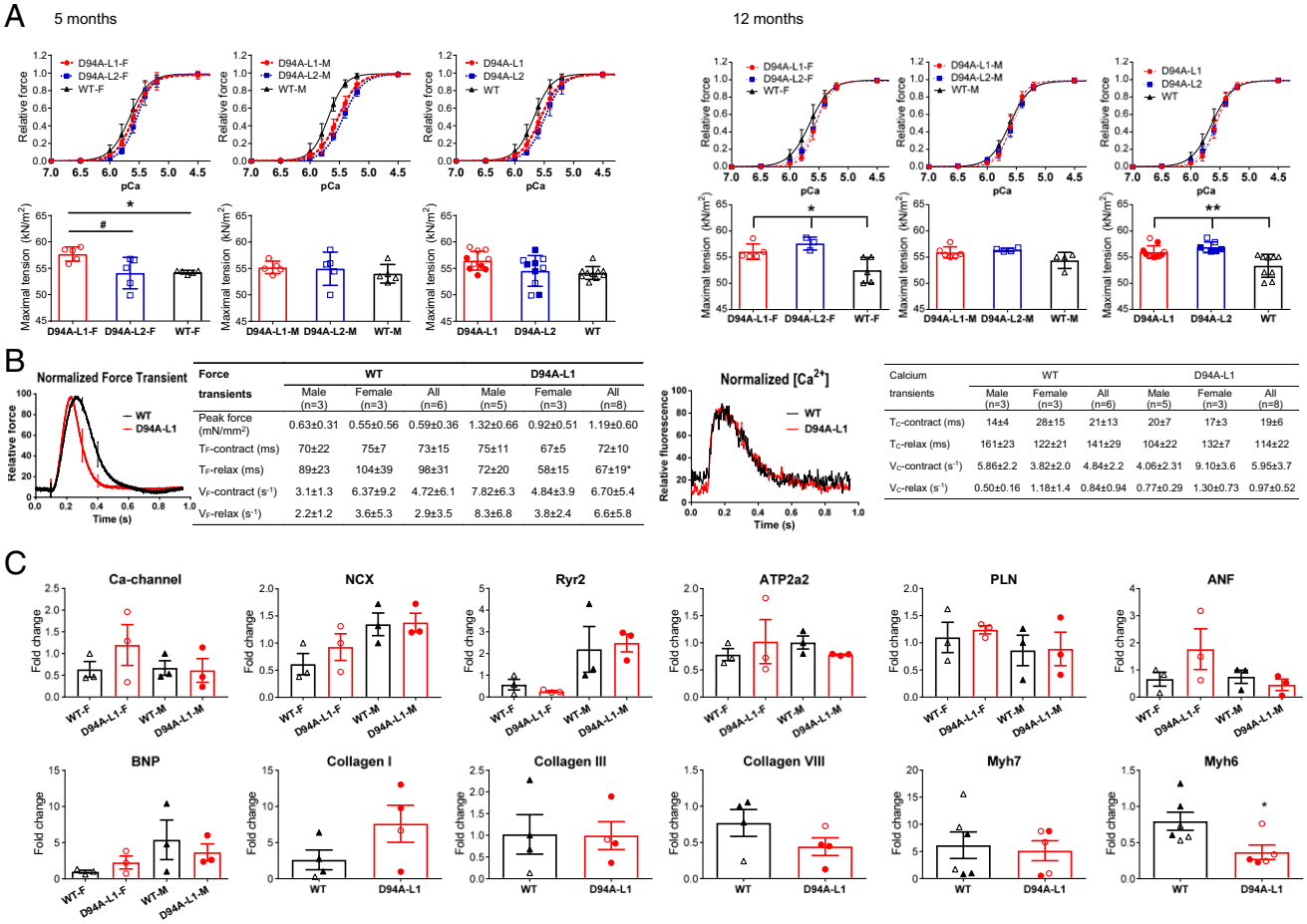
Parameter	D94A-L1-F	D94A-L2-F	WT-F	D94A-L1-M	D94A-L2-M	WT-M
Echocardiography						
No. of animals	14	6	8	9	6	10
Heart/body, mg/g	4.63 ± 0.16	4.52 ± 0.36	4.48 ± 0.29	4.66 ± 0.12	5.15 ± 0.14	5.31 ± 0.21
Heart/tibia, mg/mm	7.01 ± 0.2	6.8 ± 0.19	6.69 ± 0.55	9.06 ± 0.27	10.34 ± 0.35*	8.74 ± 0.3[a]
HR, bpm	503 ± 9	498 ± 17	478 ± 17	512 ± 15	520 ± 11	497 ± 14
IVCT, ms	15.09 ± 1.01	12.48 ± 1.72	15.33 ± 1.6	15.49 ± 1.41	11.61 ± 1.11	15.09 ± 1.6
IVRT, ms	17.06 ± 1.5	14.92 ± 1.42	15.85 ± 0.9	15.76 ± 1.59	13.94 ± 0.46	14 ± 0.69
MV E/A	2.63 ± 0.24	1.83 ± 0.23	2.4 ± 0.29	1.88 ± 0.19	1.74 ± 0.17	1.91 ± 0.13
MV E/E′	−26.7 ± 2.4	−24.2 ± 2.8	−25.9 ± 2.0	−22.1 ± 1.3	−31.2 ± 2.1	−25.5 ± 2.4
EF, %	47 ± 2**	52 ± 1	60 ± 3	35 ± 2***,#,[a]	45 ± 2**,[a]	60 ± 2
FS, %	23 ± 1*	22 ± 2*	31 ± 3	19 ± 1[a],**	23 ± 1	29 ± 2
IVS;d, mm	0.81 ± 0.03	0.76 ± 0.03	0.74 ± 0.03	0.82 ± 0.06	0.87 ± 0.03	0.9 ± 0.04[a]
IVS;s, mm	1.09 ± 0.04	0.98 ± 0.06	1.09 ± 0.08	1.11 ± 0.09	1.13 ± 0.05	1.21 ± 0.08
LVID;d, mm	4.05 ± 0.11	3.97 ± 0.09	3.89 ± 0.08	4.19 ± 0.12	4.42 ± 0.08[a]	4.16 ± 0.1[a]
LVID;s, mm	3.12 ± 0.12*	3.09 ± 0.09	2.66 ± 0.14	3.38 ± 0.13*	3.39 ± 0.10*	2.96 ± 0.13
LVPW;d, mm	0.7 ± 0.03	0.71 ± 0.03	0.73 ± 0.02	0.8 ± 0.05	0.77 ± 0.04	0.78 ± 0.02
LVPW;s, mm	0.9 ± 0.05*	0.96 ± 0.05	1.06 ± 0.06	0.99 ± 0.06	1.06 ± 0.07	1.14 ± 0.04
Hemodynamics						
No. of animals	10	6	5	6	5	6
SW, mmHg×μL	1,779 ± 144	1,898 ± 137	1,896 ± 127	1,741 ± 259	2,592 ± 153[a]	2,577 ± 344
CO, μL/min	11,349 ± 1,155	13,330 ± 753	12,538 ± 1,109	11,106 ± 1,138*	16,938 ± 916[a]	16,673 ± 1,777
SV, μL	23.24 ± 2.06	24.99 ± 1.14	24.92 ± 1.11	21.78 ± 1.87*,#	31.54 ± 1.57[a]	32.86 ± 2.89[a]
ESV, μL	31.92 ± 3.46	30.65 ± 1.91	23.6 ± 2.26	45.95 ± 2.56*,[a]	41.22 ± 1.94[a]	34.31 ± 3.66[a]
EDV, μL	49.25 ± 4.09	50.39 ± 2.65	44.02 ± 1.8	62.49 ± 3.51	67.98 ± 2.35[a]	59.35 ± 4.49[a]
Pes, mmHg	100.2 ± 3.03*	97.31 ± 3.78	87.13 ± 3.21	99.6 ± 4.96	95.23 ± 1.59	92.23 ± 3.61
Ped, mmHg	5.48 ± 0.78	7.22 ± 2.29	5.42 ± 1.42	5.42 ± 1.09	4.91 ± 1.72	7.29 ± 1.8
HR, bpm	484 ± 16	532 ± 8	499 ± 25	506 ± 20	537 ± 12	504 ± 13
Ea, mmHg/μL	4.54 ± 0.28*	3.94 ± 0.21	3.53 ± 0.14	4.75 ± 0.48**	3.02 ± 0.17	2.93 ± 0.3
Ea/Ees	1.73 ± 0.22	1.99 ± 0.56	0.84 ± 0.08	1.36 ± 0.25	0.96 ± 0.25	1.42 ± 0.24
dP/dt_{max}, mmHg/s	9,339 ± 787	11,099 ± 1,794	9,249 ± 574	8,818 ± 659	9,010 ± 544	8,248 ± 575
dP/dt_{min}, mmHg/s	−9,428 ± 516	−9,593 ± 1,162	−9,133 ± 405	−8,496 ± 791	−9,655 ± 622	−8,784 ± 1,051
Tau, ms	6.22 ± 0.18	6.51 ± 0.82	5.87 ± 0.29	6.44 ± 0.23	5.46 ± 0.22	6.86 ± 1.18
No. of animals	9	4	5	6	5	5
ESPVR	3.24 ± 0.47	2.9 ± 0.76	4.24 ± 0.38	3.99 ± 0.81	4.39 ± 1.4	2.35 ± 0.52
EDPVR	0.15 ± 0.02	0.14 ± 0.02	0.16 ± 0.03	0.15 ± 0.06	0.10 ± 0.02	0.10 ± 0.01
PRSW	52.53 ± 4.59	58.09 ± 6.02	70.64 ± 5.78	60.69 ± 2.6	75.14 ± 6.35	68.64 ± 5.84
dP/dt_{max}-EDV	184.62 ± 21.3	184.78 ± 35.6	150.1 ± 19.66	172.61 ± 24.6	144.58 ± 15.8	117.44 ± 19.1

Heart weight/body weight (in mg/g); heart weight/tibia length (in mg/mm); CO, cardiac output; d, diastolic; dP/dt_{max}, peak rate for pressure rise; $-dP/dt_{min}$, peak rate for pressure decline; EDPVR, slope of end-diastolic PV relationship; EDV, end-diastolic volume; Ees, end-systolic elastance, slope of ESPVR (end-systolic pressure–volume relationship); ESV, end-systolic volume; FS, fractional shortening; HR, heart rate (in beats per min); IVCT, isovolumetric contraction time; IVRT, isovolumetric relaxation time; IVS, interventricular septum; LVID, left ventricular inner diameter; LVPW, LV posterior wall; MV E/A, mitral early (E)-to-late (A) diastolic inflow velocity; E′, early diastolic velocity; Ped, end-diastolic pressure; Pes, end-systolic pressure; PRSW, prerecruitable SW (slope of SW–EDV relationship); s, systolic; SV, stroke volume; SW, stroke work; Tau, relaxation time constant. Data are the mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ D94A vs. WT, #$P < 0.05$ L1 vs. L2, by one-way ANOVA and the post hoc Tukey's multiple comparison test; [a]$P < 0.05$ M vs. F, by Student's t test.

contractile force at submaximal calcium concentrations (pCa 6 to 5). These data suggest that changes in the Ca^{2+}-dependent actin–myosin interactions trigger abnormal heart remodeling in D94A mice and result in decompensated cardiac systolic function (low EF) and dilated cardiomyopathy. Consistent with the D94A effects observed in this report, DCM-associated mutations in the thin-filament proteins studied in mice (deletion mutation ΔK210 in *TNNT2*) resulted in a diminished myofilament Ca^{2+} sensitivity with no changes in maximal contractile force (13).

We also investigated whether the changes in the force–pCa relationship observed in D94A mice originated from cellular calcium dysregulation or from abnormal myosin motor activity or other sarcomeric dysfunction. Force and calcium transients were measured in intact papillary muscle fibers from 6- to 8-mo-old D94A mice and age- and sex-matched WT mice using the IonOptix Calcium and Contractility Recording System (Fig. 4*B*). Electrically stimulated isometric twitches were recorded simultaneously with $[Ca^{2+}]$ transients after 1.5-h incubation with Fura-2 AM. No differences in peak force were found between the genotypes (Fig. 4*B*) but, consistent with the decreased myofilament Ca^{2+} sensitivity in skinned fibers, the relaxation phase of force transients was significantly faster (shorter time) in D94A compared with WT muscles (67 vs. 98 ms). No changes in intracellular $[Ca^{2+}]$ transients were observed (Fig. 4*B*). These data led us to the conclusion that the contractile properties of D94A-mutated myosin cross-bridges and their altered interaction with the thin filaments are most likely responsible for development of DCM in D94A animals.

Consistent with the lack of changes in myocellular calcium homeostasis, the gene expression profiles of the major intracellular players of the excitation–contraction coupling revealed no differences between D94A and WT hearts, and no differences were also noted between male and female mice (Fig. 4*C*). Total mRNA was isolated from the ventricles of three male and three female 5- to 6-mo-old D94A-L1 and from WT control mice. No D94A-mediated changes



Force transients	WT Male (n=3)	WT Female (n=3)	WT All (n=6)	D94A-L1 Male (n=5)	D94A-L1 Female (n=3)	D94A-L1 All (n=8)
Peak force (mN/mm²)	0.63±0.31	0.55±0.56	0.59±0.36	1.32±0.66	0.92±0.51	1.19±0.60
T_F-contract (ms)	70±22	75±7	73±15	75±11	67±5	72±10
T_F-relax (ms)	89±23	104±39	98±31	72±20	58±15	67±19*
V_F-contract (s^{-1})	3.1±1.3	6.37±9.2	4.72±6.1	7.82±6.3	4.84±3.9	6.70±5.4
V_F-relax (s^{-1})	2.2±1.2	3.6±5.3	2.9±3.5	8.3±6.8	3.8±2.4	6.6±5.8

Calcium transients	WT Male (n=3)	WT Female (n=3)	WT All (n=6)	D94A-L1 Male (n=5)	D94A-L1 Female (n=3)	D94A-L1 All (n=8)
T_C-contract (ms)	14±4	28±15	21±13	20±7	17±3	19±6
T_C-relax (ms)	161±23	122±21	141±29	104±22	132±7	114±22
V_C-contract (s^{-1})	5.86±2.2	3.82±2.0	4.84±2.2	4.06±2.31	9.10±3.6	5.95±3.7
V_C-relax (s^{-1})	0.50±0.16	1.18±1.4	0.84±0.94	0.77±0.29	1.30±0.73	0.97±0.52

Fig. 4. (*A*) Contractile function in papillary muscle fibers from ∼5- and ∼12-mo-old D94A-L1 and -L2 vs. WT mice. Open symbols depict female and closed symbols indicate male animals. (*B*) The effect of D94A mutation on force and calcium transients in intact papillary muscles. The measurements were performed on intact papillary muscles from 6- to 8-mo-old gender-matched D94A-L1 (five M and three F) and WT (three M and three F) mice. Time (in milliseconds) to depart 50% (t_{50}) from t_0 to peak during contraction, and from peak to baseline during relaxation, is characterized by T_F-contract and T_F-relax (force transients) and T_C-contract and T_C-relax ([Ca^{2+}] transients). Maximum velocity of force departing from and returning to the baseline during muscle contraction and relaxation is reported as V_F-contract and V_F-relax (force transients) and V_C-contract and V_C-relax ([Ca^{2+}] transients). Note a significant (*$P <$ 0.05) decrease in T_F-relax in D94A-L1 vs. WT fibers. Results are presented as the average ± SD of *n* animals with *$P < 0.05$ and **$P \leq 0.01$ for mutant vs. WT and $^{\#}P < 0.05$ for D94A-L1 vs. D94A-L2, using one-way ANOVA (*A*) or Student's *t* test (*B*). (*C*) Gene expression profiles in D94A and WT mice. Real-time qPCR was performed on three F (open symbols) and three M (close symbols) D94A-L1 and WT mice, and the results are presented as fold change in transcript expression in D94A vs. WT mice. Data are the mean ± SEM. *$P < 0.05$, using one-way ANOVA with the Tukey's multiple comparison test.

in the expression of voltage-dependent L-type calcium channel, alpha-1C (Ca^{2+} channel), sodium–calcium exchanger, cardiac ryanodine receptor (Ryr2), ATP2a2 (SERCA2), and phospholamban genes were observed compared with WT control hearts (Fig. 4*C*). No changes in *myh7* between genotypes were noted but, in agreement with reports on DCM patients (14), mRNA of *myh6* was significantly down-regulated in D94A animals (Fig. 4*C*). In addition, ANF and BNP, stress response genes, were slightly up-regulated in D94A-F compared with WT-F mice, but the difference between genotypes did not reach statistical significance (Fig. 4*C*).

Hypocontractility of D94A-RLC Myosin. To understand the effect of D94A on myosin motor function, actin-activated ATPase activity assays were conducted on cardiac myosin purified from ventricles of D94A and WT mice. Consistent with other DCM mutations in α-MHC motor proteins studied in mice (15), a significantly lower V_{max} was observed for D94A compared with WT myosin (Fig. 5*A*), suggesting a slower transition from the weakly (A·M·ATP ↔ A·M·ADP·Pi) to strongly (A·M·ADP ↔ A·M) bound D94A cross-bridges. No differences in the Michaelis–Menten constant (K_m) were noted between the groups. Significantly, this hypocontractile activity of D94A-mutated myosin motors reflects a diminished ability of myosin to hydrolyze ATP to produce energy for muscle contraction (Fig. 5*A*).

Fluorescence-based steady-state binding of D94A-myosin to pyrene-labeled F-actin under rigor (no ATP) conditions supports the ATPase data and shows a stronger interaction of myosin with actin for the mutant, suggesting slower myosin dissociation rates (Fig. 5*B*). Lower V_{max} of D94A-myosin could also be due to fewer myosin heads participating in the contractile cycle, and both scenarios indicate a mutation-induced hypocontractility of the myosin motor and a compromised chemomechanical cycle that contracts the sarcomere, leading to lower EF and systolic dysfunction in the D94A mouse model of DCM.

Small-Angle X-Ray Diffraction Patterns in D94A and WT Papillary Muscles. The interfilament lattice spacing ($d_{1,0}$) can be used to derive the center-to-center distance between two adjacent thick and thin filaments. The intensity ratio ($I_{1,1}/I_{1,0}$) is the ratio of the integrated intensity of the 1,1 equatorial reflection (representing density in the plane containing both thick and thin filaments) to that of the 1,0 equatorial reflection (reflecting density in the

Table 2. Contractile function in skinned papillary muscle fibers from ∼5- and ∼12-mo-old D94A-RLC mice compared with WT-RLC animals

Parameter	D94A-L1-F	D94A-L2-F	WT-F	D94A-L1-M	D94A-L2-M	WT-M	D94A-L1$_{ALL}$	D94A-L2$_{ALL}$	WT$_{ALL}$
Five-mo-old mice									
No. of animals (fibers)	5 (40)	5 (46)	5 (39)	5 (38)	5 (39)	5 (49)	10 (78)	10 (85)	10 (88)
F_{max}, kN/m^2	57.71 ± 1.36$^{*,\#}$	54.1 ± 2.98	54.27 ± 0.32	55.19 ± 1.18	54.94 ± 3.14	53.99 ± 1.76	56.45 ± 1.79	54.52 ± 2.92	54.13 ± 1.2
pCa$_{50}$	5.56 ± 0.08	5.54 ± 0.05*	5.67 ± 0.09	5.52 ± 0.06$^{**,\#}$	5.44 ± 0.01**	5.69 ± 0.07	5.54 ± 0.07**	5.49 ± 0.06**	5.68 ± 0.07
Hill coefficient	2.99 ± 0.68	3.61 ± 0.88	2.81 ± 0.29	2.8 ± 0.82	2.89 ± 0.61	3.25 ± 0.78	2.89 ± 0.72	3.25 ± 0.81	3.03 ± 0.6
Twelve-mo-old mice									
No. of animals (fibers)	5 (44)	3 (18)	5 (46)	5 (44)	4 (19)	4 (51)	11 (88)	7 (37)	9 (97)
F_{max}, kN/m^2	56.06 ± 1.47*	57.62 ± 1.22*	52.51 ± 2.45	55.89 ± 1.07	56.39 ± 0.35	54.38 ± 1.54	55.97 ± 1.2*	56.91 ± 0.99*	53.34 ± 2.21
pCa$_{50}$	5.55 ± 0.05	5.56 ± 0.01	5.66 ± 0.1	5.58 ± 0.06	5.56 ± 0.04	5.61 ± 0.05	5.57 ± 0.05*	5.56 ± 0.03*	5.64 ± 0.08
Hill coefficient	3.78 ± 0.35$^{**,\#\#}$	2.84 ± 0.25	2.65 ± 0.19	3.8 ± 0.29$^{**,\#\#}$	2.97 ± 0.45	2.71 ± 0.21	3.79 ± 0.3$^{**,\#\#}$	2.91 ± 0.36	2.68 ± 0.19

F_{max}, tension per cross-section of muscle; pCa$_{50}$, calcium concentration for half-maximal activation (Ca^{2+} sensitivity). Data are the mean ± SD of n (number of animals) experiments; $^*P < 0.05$ and $^{**}P \leq 0.01$ for mutant vs. WT, and $^{\#}P < 0.05$ and $^{\#\#}P \leq 0.01$ for D94A-L1 vs. D94A-L2, using one-way ANOVA.

plane containing only thick filaments) (16). Both parameters ($d_{1,0}$ and $I_{1,1}/I_{1,0}$) are important metrics of sarcomeric structure that were previously shown to be sensitive to amino acid substitutions in any of the sarcomeric components (10, 17).

The equatorial X-ray patterns were collected simultaneously with the measurements of force at pCa 8 to 4 on skinned papillary muscles from D94A versus WT mice at sarcomere length 2.1 μm (10). While no changes due to the D94A mutation were found in $d_{1,0}$ (in nm), there was a significant decrease in $I_{1,1}/I_{1,0}$ ratio upon fiber activation (Fig. 6). The smaller $I_{1,1}/I_{1,0}$ in DCM versus WT mice indicates repositioning of the D94A-myosin cross-bridge mass closer to the thick-filament backbone (10, 17). We propose that this reduced $I_{1,1}/I_{1,0}$ ratio in D94A mice reflects less association of the myosin heads with the thin filaments and underlies the key structural change in the sarcomere that results in desensitization of myofilaments to Ca^{2+}. Consistent with this line of thinking, a significantly reduced Ca^{2+} sensitivity of force was measured in skinned papillary muscle fibers from D94A mutant versus WT mice (Fig. 4*A*). On the other hand, in accordance with no changes in maximal force developed by D94A muscle fibers (Fig. 4 *A* and *B*), no changes in the interfilament lattice spacing, $d_{1,0}$, was measured upon activation (at pCa 5.2) in D94A mice compared with WT (Fig. 6).

Discussion

Dilated cardiomyopathy is a severe pathology of the heart that can appear as a spectrum of symptoms, from subtle to severe, including congestive heart failure. Reduced contractile function and pathological remodeling of the heart are recognized clinical hallmarks of DCM, but the early molecular events that impair heart performance are unknown and require further investigation. Consistent with DCM characteristics in human patients carrying the D94A mutation in MYL2 (5), the D94A transgenic mouse model presents with ventricular chamber dilation, which is accompanied by a decreased ejection fraction and mild global left ventricular systolic dysfunction (Fig. 3). Although the histologic alterations include interstitial and replacement fibrosis, D94A animals do not demonstrate excessive fibrotic lesions and only mild fibrosis is observed in older D94A male hearts (Fig. 2). This result is consistent with findings of other studies on small or large animal models of DCM suggesting that myocyte hypertrophy and fibrosis are not prominent features in this form of cardiomyopathy despite changes in hemodynamics and LV chamber structure and function (15, 18).

In addition to occurrences of fibrosis in male and not female D94A hearts (Fig. 2), the diminished cardiac function was also observed to be more severe in male than in female D94A mice

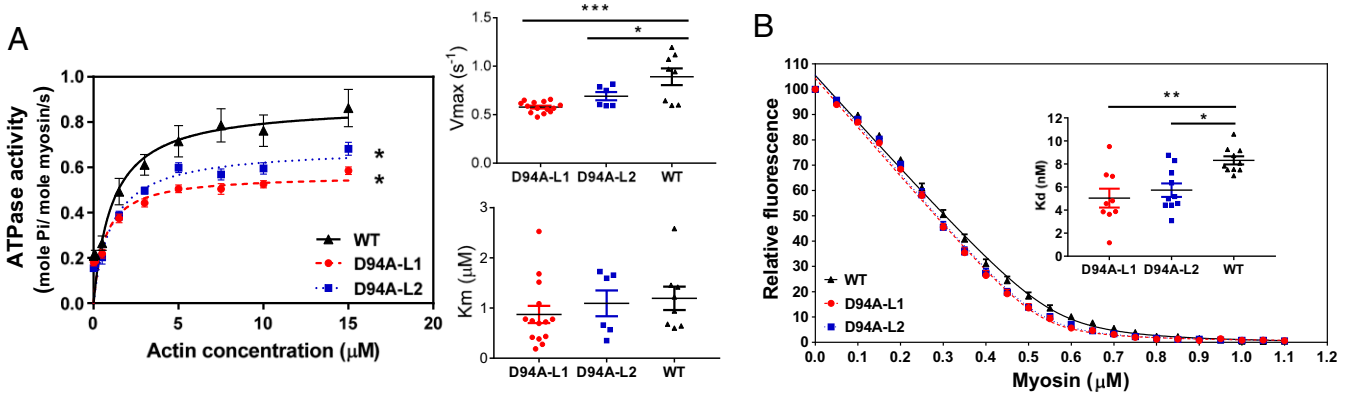
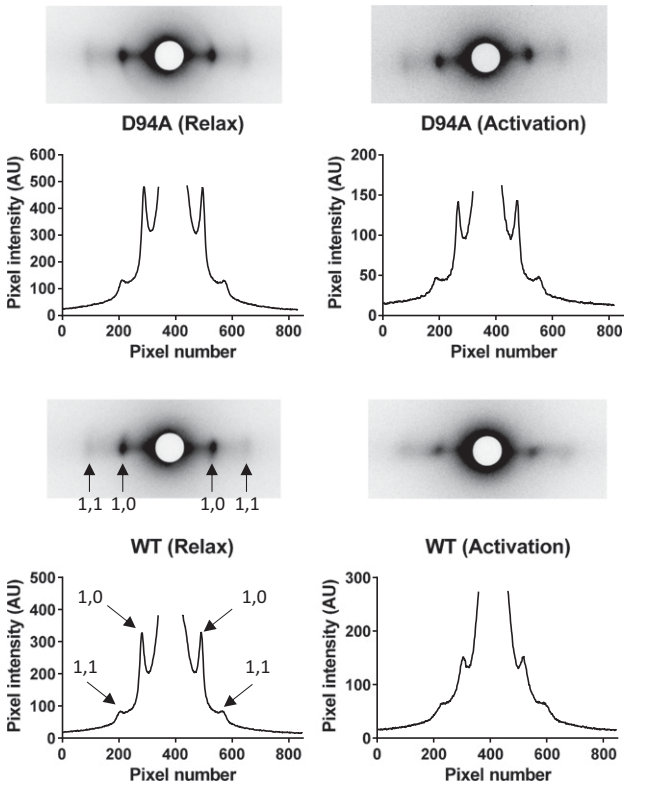


Fig. 5. (*A*) Actin-activated myosin ATPase activity of D94A and WT myosins. A significantly lower V_{max} was observed for D94A-L1 (0.57 ± 0.02 s^{-1}; $n = 14$) and D94A-L2 (0.69 ± 0.03 s^{-1}; $n = 6$) myosins compared with WT (0.88 ± 0.05 s^{-1}; $n = 8$). (*B*) Fluorescence-based binding assays of D94A or WT myosin to pyrene-labeled F-actin in the absence of ATP (rigor). Note a significantly higher binding affinity of D94A-L1 (K_d 4.48 ± 0.67 nM; $n = 9$) and D94A-L2 myosin (K_d 5.73 ± 0.58 nM; $n = 10$) vs. WT (K_d 8.31 ± 0.35 nM; $n = 10$) control. Both assays (*A* and *B*) were performed on myosin purified from left and right ventricles of 5- to 8-mo-old F and M D94A and WT mice. Approximately four or five hearts per group were used to generate one batch of myosin, and the assays were repeated with two to four different batches of myosin. No differences between the sexes were noted, and the data from M and F mice were pooled. Data are the mean ± SEM of n experiments with $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ calculated, using one-way ANOVA with the Tukey's multiple comparison test.



	WT	Nº fibers	D94A-L1	Nº fibers	P-value
Intensities ratio ($I_{1,1}/I_{1,0}$)					
pCa 8 (Relaxation)	0.22±0.06	15 (7F, 8M)	0.26±0.08	15 (8F, 7M)	0.206
pCa 5.2 (Activation)	0.43±0.02	14 (7F, 7M)	0.35±0.03*	12 (4F, 8M)	0.043
Lattice spacing ($d_{1,0}$ in nm)					
pCa 8 (Relaxation)	41.54±0.3	16 (8F, 8M)	41.38±0.24	15 (8F, 7M)	0.722
pCa 5.2 (Activation)	41.45±0.31	15 (7F, 8M)	41.64±0.29	14 (7F, 7M)	0.701

Fig. 6. Small-angle X-ray diffraction patterns and integrated intensity traces collected from papillary muscles from D94A-L1 (n = 3 F and 3 M mice) vs. WT (n = 4 F and 3 M mice) in relaxation (pCa 8) and activation (pCa 5.2). All patterns show clearly resolved 1,0 and 1,1 reflections. Note the significant difference in $I_{1,1}/I_{1,0}$ between D94A-L1 and WT (P = 0.043). Data are the mean ± SEM of n animals with *P < 0.05, using Student's t test.

(Fig. 3). A recent study has shown that in all cardiomyopathy subtypes there was an unequal sex distribution, with a tendency toward men for DCM, HCM (hypertrophic cardiomyopathy), and ARVC (arrhythmogenic right ventricular cardiomyopathy) and toward women for RCM (restrictive cardiomyopathy) (19). Reports also show that under disease conditions, females have higher expression of genes related to energy metabolism and can better maintain their metabolic function in response to a disease stimulus than males (20). Furthermore, significant sex differences in genes involved in the regulation of fibrosis and inflammation with a significant repression of these processes in women were also reported (21), indicating that females may be protected against effects of fibrosis and inflammation.

Previous studies have implicated different cytoskeletal and sarcomeric proteins in familial DCM, but the mechanisms by which these divergent mutations cause disease range from impairment of force transduction (22, 23) and disturbance of myocellular Ca^{2+} (24) to direct effects of sarcomeric protein mutations on myofilament Ca^{2+} sensitivity and contractile force generation, leading to systolic dysfunction and DCM (25). Creation of genetically altered transgenic and knockin mice expressing mutant proteins in their hearts provides valuable animal models to study the molecular mechanisms of DCM pathogenesis (13, 15, 26–29). Consistent with our results, mouse models of two MYH7 mutations (S532P and F764L) in an α-MHC background displayed depressed contractile function in isolated cardiomyocytes and a reduced ability of mutant myosins to translocate actin (V_{actin}) but had similar force-generating capacities (15). Thin-filament mouse models of DCM carrying disease-causing mutations in α-Tm (28) or troponin subunits (13, 29) demonstrated a largely reduced Ca^{2+} sensitivity and thus caused impairment of cardiac function through a malfunction of the Tm–Tn regulatory system. On the other hand, a knockin mouse model of DCM expressing a truncated form of MyBP-C (26) exhibited neonatal onset of a progressive DCM with myofibrillar disarray, fibrosis, and dystrophic calcification, but increased oxidative stress and myocardial inflammation were thought to underlie the diminished contractile response and heart failure in these mice (27, 30).

Small-angle X-ray diffraction patterns in skinned papillary muscles from D94A mice revealed distinct structural abnormalities in the sarcomeres of mouse hearts that may explain the underlying DCM phenotype. We demonstrate here that structural consequences of the D94A mutation in myosin RLC (Fig. 6) closely correlate with the functional abnormalities in D94A papillary muscle fibers (Table 2) and with abnormal heart function in echocardiography and invasive hemodynamics experiments (Table 1). The ability of D94A-mutated myosin to generate contractile force in skinned papillary muscle fibers at a level comparable to WT mice (Fig. 4*A*) correlated well with no changes in the interfilament lattice spacing ($d_{1,0}$) measured in D94A and WT fibers (Fig. 6). On the other hand, consistent with the reduced Ca^{2+} sensitivity of force observed in D94A fibers (Fig. 4*A*), a statistically significant decrease in $I_{1,1}/I_{1,0}$ ratio was measured in D94A fibers upon submaximal activation compared with WT (Fig. 6). A decrease in $I_{1,1}/I_{1,0}$ represents less association of the myosin heads with the thin filaments, which could explain the rightward shift in the force–pCa relationship in D94A versus WT fibers. Therefore, the structural changes in the D94A-RLC–mutated myosin motors stabilized their hypocontractile conformation (31) and led to desensitization of the myofilaments to calcium during force generation. These changes ultimately resulted in reduced ejection fraction and systolic abnormalities in D94A hearts.

The molecular pathogenesis of dilated cardiomyopathy was recently assessed in the context of the myosin superrelaxed state and interacting-heads motif (IHM) paradigm (31). Similar to the effects seen in this report (Fig. 5*A* and Table 2), DCM-causing mutations in MYH7 were shown to have modest effects on IHM interactions but have substantially reduced MHC motor functions, particularly nucleotide binding, resulting in reduced ATP consumption and decreased contractility. The authors elegantly demonstrated that genetic DCM and HCM variants differentially impact myosin motor functions and specific IHM interactions explaining the mechanisms for diminished contractile power in DCM and the distinctly compromised relaxation and energetics in HCM (31). Our data suggest that the D94A-RLC mouse model may be highly suitable for testing of novel myosin-specific therapeutics for systolic heart dysfunction, for example, omecamtiv mecarbil, aimed at increasing cardiac myosin activation and the duration of ejection without changing cardiomyocyte calcium homeostasis (32).

The results of this study strongly suggest that mice carrying the D94A-RLC mutation develop dilated cardiomyopathy by a mechanism involving direct effects of D94A on cardiac myosin, the molecular motor of the heart that transduces chemical energy from ATP hydrolysis into mechanical energy to drive cardiac

muscle contraction and support heart pump function to meet body demands for blood. Supporting this notion, the gene expression profiles of the major players in excitation–contraction coupling showed no differences between D94A and WT hearts (Fig. 4*C*). Studies on animal models of cardiomyopathy and human heart failure samples argue that dysregulation of RLC phosphorylation plays a role in the manifestation of the disease phenotype (10, 33, 34). However, in our previous in vitro study (5), as well as in this report, the D94A mutation did not reduce the overall RLC phosphorylation in the mutant myocardium compared with WT mice (Fig. 1*C*). Perhaps, as proposed by the Ferenczi group (35), the steady-state level of RLC phosphorylation in D94A mice may signify a compensatory adaptation to DCM pathology.

To conclude, D94A-mediated DCM is a rather complex issue, but clearly involves abnormal interactions between the hypocontractile myosin motors and actin–Tm–Tn filaments. The structural perturbations located at the level of sarcomeres result in aberrant cardiomyocyte cytoarchitecture leading to LV chamber dilation and decreased ejection fraction manifesting in systolic dysfunction of D94A hearts. Our comprehensive study demonstrates that this novel DCM MYL2 variant with the D94A mutation in the myosin RLC follows the clinical DCM phenotype, and suggests that in addition to other sarcomeric genes, MYL2 may serve as a therapeutic target for dilated cardiomyopathy disease.

Materials and Methods

Detailed materials and methods are outlined in *SI Materials and Methods*

This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (36). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Miami Miller School of Medicine (assurance number A-3224-01, effective November 24, 2015). Euthanasia of mice was achieved through inhalation of CO_2 followed by cervical dislocation.

Generation of Transgenic DCM D94A-RLC Mice. The cDNA of human D94A-mutated ventricular RLC [National Center for Biotechnology Information (NCBI) accession no. NP_000423.2] was inserted downstream of the α-MHC promoter (clone 26, provided by Jeffrey Robbins, Cincinnati Children's Hospital Medical Center, Cincinnati, OH), and two Tg lines (D94A-L1 and D94A-L2) expressing mutated human RLC in mice were produced as described previously (8). The results were compared with previously produced Tg-WT mice expressing nonmutated human ventricular RLC (8). Relative expression of D94A mutant RLC in Tg lines was assessed by mass spectrometry (MS). SDS/PAGE bands of mouse purified myosin from four or five hearts per group were digested with trypsin and then reduced and alkylated. Digested peptides were analyzed by MS, and protein identification was determined using X! Tandem (9). Relative amounts of human versus mouse protein were determined by the ratio of the number of human-specific peptides (AGGANSN or EEVDQM) or mouse-specific peptides (IEGGSSN or EEIDQM) for which the most identified spectra were obtained (9, 37).

Heart Morphology and Function in D94A and WT Animals. Histopathology examination of paraffin-embedded longitudinal sections of whole mouse hearts stained with hematoxylin and eosin and Masson's trichrome, and the electron microscopy evaluation of myocardial ultrastructure, was assessed as described earlier (9, 10). Heart morphology and function were examined in male and female ∼5- and ∼12-mo-old D94A mouse lines (L1 and L2) and compared with age- and gender-matched WT mice by echocardiography and invasive hemodynamic assessments as outlined in detail in Yuan et al. (9, 10).

Myosin Cross-Bridge Contractile and Structural Properties in Skinned and Intact Fibers from D94A Versus WT Mice. Binding of D94A mouse purified myosin to pyrene–actin and actin-activated myosin ATPase activity assays were performed as previously reported (9, 10). Steady-state force development and force–pCa measurements were performed on skinned papillary muscle fibers with sarcomere length adjusted to 2.1 μm, according to previously described protocols (9). Approximately 5- and 12-mo-old D94A-L1 and -L2 animals were used with each heart, yielding on average 8 to 10 individual muscle strips. Force and [Ca^{2+}] transient measurements were performed on intact papillary muscles from 6- to 8-mo-old sex-matched D94A-L1 (five M and three F) and WT (three M and three F) mice using the IonOptix Calcium and Contractility Recording System. Intact muscle fibers were stimulated at 1 Hz, and force and fluorescence (340/380 nm ratio) transients were recorded (8). The equatorial X-ray patterns were collected simultaneously with the measurements of force at pCa 8 to 4 on skinned papillary muscles from D94A-L1 (three F and three M) versus WT-RLC (four F and three M) mice at sarcomere length 2.1 μm (10).

Statistical Analysis. All values are shown as means ± SD or means ± SEM. Statistically significant differences between two groups were determined using an unpaired Student's *t* test, with significance defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Comparisons between multiple groups were performed using one-way ANOVA and the Tukey's multiple comparison test.

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